Using cysteine to solve hemolysis effect issues

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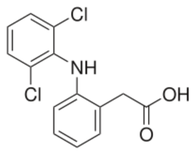
Introduction



MW: 296.1 amu

Hemolysis can be defined as the rupturing of erythrocytes (red blood cells) and the release of their contents (cytoplasm) into surrounding fluid (e.g., plasma). It may occur in vivo or in vitro. During bioanalytical studies, it may occur during the sample collection handling and procedure. Therefore, its impact on the quantitation must be adequately determined during the matrix effect evaluation. In this case study, cysteine was used to solve an hemolysis effect issue observed during the method development of diclofenac in human plasma.

Method

Diclofenac is extracted by an automated liquid-liquid extraction using a pH 2 buffer and MTBE. Analysis was performed using an API5000 equipped with an ACE C18 column. Plasma was spiked with 2 and 5% hemolyzed whole blood. Low and high hemolyzed quality controls were analyzed with a fresh calibration curve. Cysteine, ascorbic acid and EDTA were tested as potential inhibiters of the hemolysis effect during the extraction of diclofenac in human plasma. Being the most effective, 100 µL of cysteine is now added to the plasma samples during sample extraction.

Extraction Procedure

- Internal Standard: Diclofenac-d_4
- Sample Volume: 50 µL
- Extraction Type: Automated liquid-liquid extraction with MTBE
- Concentration factor: 0.025

LC-MS/MS Analysis

Chromatographic mode:	Reversed Phase
Analytical Column:	ACE C18
Column Temperature:	Room Temperature
Elution mode:	Isocratic
Mobile Phase A:	Milli-Q Type Water/Methanol (30/70), Ammonium formate 1mM, Formic Acid 0.1%
Flow Rate:	1.0 mL/minute
Retention Times:	1.86 minutes
Acquisition time:	4.00 minutes
Detector:	AB Sciex API 4000
Source:	TurboIonSpray, Positive mode
Masses:	296 → 214 amu

Results

The diclofenac dynamic range was 5 to 5000 ng/mL. When diclofenac was determined in 5% hemolyzed quality controls at 15 and 3750 ng/mL, suppression between 20 and 50% of the analyte and internal standard responses was observed at both concentrations **(Table 1)**.

Using ascorbic acid, suppression of 20% of the analyte and internal standard responses was still observed. EDTA added to the samples in the extraction procedure did not resolve the suppression of the analyte and internal standard. Cysteine was found to inhibit the matrix effect caused by hemolysis **(Table 2)**.

Table 1. Impact of 5% Hemolysis on Quality Controls

Quality Control	Diclofenac Peak Area	Internal Standard Peak Area	Analyte/IS Ratio
Low QC 15 ng/mL	4958	81001	0.061
Hemolyzed Low QC 15 ng/mL	2473	43495	0.056
High QC 3750 ng/mL	1168405	79778	14.629
Hemolyzed High QC 3750 ng/mL	690385	49294	14.295

Table 2. Comparison of Antioxidants Impact on Hemolysed Low Quality Control (15 ng/mL)

Antioxidants	Diclofenac Peak Area	Internal Standard Peak Area	Analyte/IS Ratio
Un-Hemolyzed QC	6415	105192	0.061
Without Additive	3777	68919	0.055
Cysteine 1%	5391	95564	0.056
Cysteine 0.5%	4268	78782	0.054
Ascorbic Acid	4284	78977	0.054
EDTA	4649	80065	0.058
Without Additive with a different Lot of Blood	3974	69873	0.057

Cysteine 1%(v/v) was added to the sample extraction at the beginning of the procedure in a proportion of 2:1 cysteine:plasma. The method was validated according the most recent validation guidances. Matrix effect including hemolysed as well as lipemic plasma was evaluated successfully. Accuracy and precision was also demonstrated.

Table 3. Matrix Effect at Low Quality Control Level (15 ng/mL)

Untreated Standard (MFQC1)		Reference Solution (RSQC1)				
Analyte Responses	Internal Standard Responses	Analyte Responses	Internal Standard Responses	Calculated Matrix Factor (Analyte)	Calculated Matrix Factor (IS)	IS-Normalized Matrix Factor
12083	415013	11676	421204	1.0560	1.0047	1.0511
11438	414392	11679	424216	0.9997	1.0032	0.9965
11485	415502	11275	412919	1.0038	1.0058	0.9979
11820	410120	10996	407838	1.0330	0.9928	1.0405
11760	415526	11452	406144	1.0278	1.0059	1.0218
12019	416141	11574	406217	1.0504	1.0074	1.0427
Mean		**11442**	**413090**			**1.0251**
SD(±)						**0.0236**
CV(%)						**2.31**

Table 4. Matrix Effect at High Quality Control Level (5000 ng/mL)

Untreated Standard (MFULOQ)		Reference Solution (RSULOQ)				
Analyte Responses	Internal Standard Responses	Analyte Responses	Internal Standard Responses	Calculated Matrix Factor (Analyte)	Calculated Matrix Factor (IS)	IS-Normalized Matrix Factor
3844302	395123	3717994	386052	1.0155	1.0152	1.0003
3765687	384692	3768275	385221	0.9947	0.9884	1.0064
3730065	385125	3821107	394497	0.9853	0.9895	0.9957
3692396	375620	3807341	390350	0.9754	0.9651	1.0106
3699494	376103	3830116	392933	0.9772	0.9664	1.0113
3696155	378568	3769127	386139	0.9764	0.9727	1.0038
Mean		**3785660**	**389199**			**1.0047**
SD(±)						**0.0060**
CV(%)						**0.60**

Table 5. Validation of 5% Hemolysis and Lipemic Effect

Quality Control	Type of Matrix	Diclofenac Area	IS Area	Analyte/IS Ratio	% Bias	Precision (%)
Low Quality Control (15 ng/mL)	Normal	7295	251544	0.0290	8.27	0.97
		8089	275054	0.0294	9.80	
		7916	267874	0.0296	10.33	
High Quality Control (3750 ng/mL)	Normal	1673213	249193	6.7145	0.12	1.03
		1778825	270383	6.5789	-1.90	
		1827808	275703	6.6296	-1.15	
Low Quality Control (15 ng/mL)	Hemolyzed	7988	285764	0.0280	9.59	2.66
		8104	289352	0.0280	9.80	
		7606	284746	0.0267	4.69	
Low Quality Control (15 ng/mL)	Lipemic	8684	315306	0.0275	2.80	3.60
		8428	291392	0.0289	8.00	
		8288	307223	0.0270	0.73	
High Quality Control (3750 ng/mL)	Hemolyzed	1815592	297190	6.1092	-4.35	3.40
		1921896	297348	6.4635	1.19	
		1672170	274725	6.0867	-4.70	
High Quality Control (3750 ng/mL)	Lipemic	2082706	305212	6.8238	1.75	0.82
		1975723	292149	6.7627	0.84	
		2062044	307143	6.7136	0.11	

Chromatography

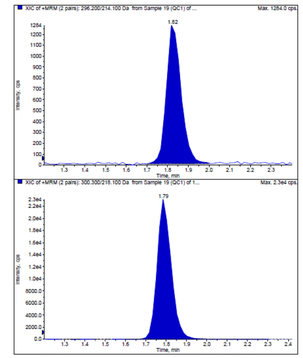


Figure 1. Representative Chromatogram of Low Quality Control (15 ng/mL) in Normal Plasma

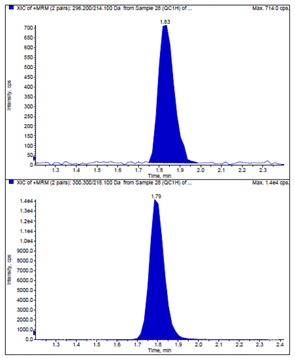


Figure 2. Representative Chromatogram of Low Quality Control (15 ng/mL) in Hemolyzed Plasma

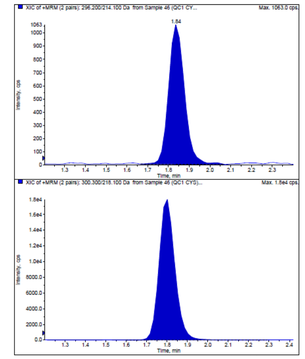


Figure 3. Representative Chromatogram of Low Quality Control (15 ng/mL) in Hemolyzed Plasma with Cysteine 1%

Conclusion

Cysteine was found to be the most effective to counter the hemolysis effect observed during the bioanalysis of diclofenac in human plasma. Cysteine 1% is added to the plasma during sample processing in a 2:1 proportion. The method was successfully used for study sample analysis. No effect caused by hemolyzed samples was observed.

TRANSFORMING PROMISING IDEAS INTO COMMERCIAL REALITY